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(54) Title: HYBRID OLIGONUCLEOTIDE PHOSPHOROTHIOATES

(57) Abstract

The invention provides hybrid oligonucleotides having phosphorothioate or phosphorodithioate internucleotide linkages, and both deoxyribonucleosides and ribonucleosides or 2'-substituted ribonucleosides. Such hybrid oligonucleotides have superior properties of duplex formation with RNA, nuclease resistance, and RNase H activation.

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HYBRID OLIGONUCLEOTIDE PHOSPHOROTHIOATES

BACKGROUND OF THE INVENTION

Field of The Invention

The invention relates to synthetic oligonucleotides that are useful for studies of gene expression and in the antisense oligonucleotide therapeutic approach. More particularly, the invention relates to synthetic oligonucleotides that have improved qualities for such applications resulting from modifications in the sugar phosphate backbone of the oligonucleotides.

Summary of The Related Art

The potential for the development of an antisense oligonucleotide therapeutic approach was first suggested in three articles published in 1977 and 1978. Paterson et al., Proc. Natl. Acad. Sci. USA 74: 4370-4374 (1987) discloses that cell-free translation of mRNA can be inhibited by the binding of an oligonucleotide complementary to the mRNA. Zamecnik and Stephenson, Proc. Natl. Acad. Sci. USA 75: 280-284 and 285-288 (1978) discloses that a 13-mer synthetic oligonucleotide that is complementary to a part of the Rous sarcoma virus (RSV) genome inhibits RSV replication in infected chicken fibroblasts and inhibits RSV-mediated transformation of primary chick fibroblasts into malignant sarcoma cells.

These early indications that synthetic oligonucleotides can be used to inhibit virus propagation and neoplasia have been followed by the use of synthetic oligonucleotides to inhibit a wide variety of viruses. Goodchild et al., U.S. Patent No. 4,806,463 (the teachings of which are hereby incorporated by reference) discloses inhibition of Human immunodeficiency virus (HIV) by synthetic oligodeoxynucleotides complementary to various regions of the HIV genome. Leiter et al., Proc.

Natl. Acad. Sci. USA 87: 3430-3434 (1990) discloses inhibition of influenza virus by synthetic oligonucleotides. Agris et al., Biochemistry 25: 6268-6275 (1986) discloses the use of synthetic oligonucleotides to inhibit Vesicular stomatitis virus (VSV). Gao et al., Antimicrob. Agents Chem. 34: 808-812 (1990) discloses inhibition of Herpes simplex virus by synthetic oligonucleotides. Birg et al., Nucleic Acids Res. 18: 2901-2908 (1990) discloses inhibition of Simian virus (SV40) by synthetic oligonucleotides. Storey et al., Nucleic Acids Res. 19: 4109-4114 (1991) discloses inhibition of Human papilloma virus (HPV) by synthetic oligonucleotides. The use of synthetic oligonucleotides and their analogs as antiviral agents has recently been extensively reviewed by Agrawal, Tibtech 10: 152-158 (1992).

In addition, synthetic oligonucleotides have been used to inhibit a variety of non-viral pathogens, as well as to selectively inhibit the expression of certain cellular genes.

Thus, the utility of synthetic oligonucleotides as agents to inhibit virus propagation, propagation of non-viral pathogens and selective expression of cellular genes has been well established. However, there is a need for improved oligonucleotides that have greater efficacy in inhibiting such viruses, pathogens and selective gene expression. Various investigators have attempted to meet this need by preparing and testing oligonucleotides having modifications in their internucleotide linkages. Several investigations have shown that such modified oligonucleotides are more effective than their unmodified counterparts. Sarin et al., Proc. Natl. Acad. Sci. USA 85: 7448-7451 (1988) teaches that oligodeoxynucleoside methylphosphonates are more active as inhibitors of HIV-1 than conventional oligodeoxynucleotides. Agrawal et al., Proc. Natl. Acad.

Sci. USA 85: 7079-7083 (1988) teaches that oligonucleotide phosphorothioates and various oligonucleotide phosphoramidates are more effective at inhibiting HIV-1 than conventional oligodeoxynucleotides. Agrawal et al., Proc. Natl. Acad. Sci. USA 86: 7790-7794 (1989) discloses the advantage of oligonucleotide phosphorothioates in inhibiting HIV-1 in early and chronically infected cells.

In addition, chimeric oligonucleotides having more than one type of internucleotide linkage within the oligonucleotide have been developed. Chimeric oligonucleotides contain deoxyribonucleosides only, but have regions containing different internucleotide linkages. Pederson et al., U.S. Patent No. 5,XXX,XXX (Ser. No. 07/480,269; allowed on 12/24/91), the teachings of which are hereby incorporated by reference, discloses chimeric oligonucleotides having an oligonucleotide phosphodiester or oligonucleotide phosphorothioate core sequence flanked by oligonucleotide phosphoramidates, methylphosphonates or phosphoramidates. Furdon et al., Nucleic Acids Res. 17: 9193-9204 (1989) discloses chimeric oligonucleotides having regions of oligonucleotide phosphodiesters in addition to either oligonucleotide phosphorothioate or methylphosphonate regions. Quartin et al., Nucleic Acids Res. 17: 7523-7562 (1989) discloses chimeric oligonucleotides having regions of oligonucleotide phosphodiesters and oligonucleotide methylphosphonates. Each of the above compounds uses deoxyribonucleotide phosphorothioates,which have reduced duplex stability. Atabekov et al., FEBS Letters 232: 96-98 (1988) discloses chimeric oligonucleotides in which all internucleotide linkages are phosphodiester linkages, but in which regions of oligoribonucleotides and oligodeoxyribonucleotides are mixed. Inoune et al., FEBS Letters, 215: 237-250 (1987) discloses chimeric

oligonucleotides having only phosphodiester linkages, and regions of oligodeoxyribonucleotides and 2'-OMe-ribonucleotides. None of these compounds having solely phosphodiester linkages exhibit either endonuclease or exonuclease resistance.

Many of these modified oligonucleotides have contributed to improving the potential efficacy of the antisense oligonucleotide therapeutic approach. However, certain deficiencies remain in the known oligonucleotides, and these deficiencies can limit the effectiveness of such oligonucleotides as therapeutic agents. Wickstrom, J. Biochem. Biophys. Methods 13: 97-102 (1986) teaches that oligonucleotide phosphodiesters are susceptible to nuclease-mediated degradation. Such nuclease susceptibility can limit the bioavailability of oligonucleotides in vivo. Agrawal et al., Proc. Natl. Acad. Sci. USA 87: 1401-1405 (1990) teaches that oligonucleotide phosphoramidates or methylphosphonates when hybridized to RNA do not activate RNase H, the activation of which can be important to the function of antisense oligonucleotides. Agrawal et al., Nucleosides & Nucleotides 8: 5-6 (1989) teaches that oligodeoxyribonucleotide phosphorothioates have reduced duplex stability when hybridized to RNA.

There is, therefore, a need for improved oligonucleotides that overcome the deficiencies of oligonucleotides that are known in the art. Ideally, such oligonucleotides should be resistant to nucleolytic degradation, should form stable duplexes with RNA, and should activate RNase H when hybridized with RNA.

BRIEF SUMMARY OF THE INVENTION

The invention provides hybrid oligonucleotides that resist nucleolytic degradation, form stable duplexes with RNA or DNA, and activate RNase H when hybridized with RNA. Oligonucleotides according to the invention provide these features by having phosphorothioate and/or phosphorodithioate internucleotide linkages and segments of oligodeoxyribonucleotides as well as segments of either oligoribonucleotides or 2'-substituted-oligoribonucleotides. For purposes of the invention, the term "2'-substituted" means substitution of the 2'-OH of the ribose molecule with, e.g, 2'-OMe, 2'-allyl, 2'-aryl, 2'-alkyl, 2'-halo, or 2'-amino, but not with 2'-H, wherein allyl, aryl, or alkyl groups may be unsubstituted or substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl or amino groups.

An object of the invention is to provide oligonucleotides that can be used to analyze and explain the importance to the effectiveness of antisense oligonucleotides of the parameters of nuclease resistance, duplex stability and RNase H activation. Another object of the invention is to provide oligonucleotides that are effective for regulating cellular, pathogen, or viral gene expression at the mRNA level. Yet another object of the invention is to provide therapeutic oligonucleotides that have great efficacy in the antisense oligonucleotide therapeutic approach. Oligonucleotides according to the invention are useful in satisfying each of these objects of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows ion exchange HPLC analysis of nuclease treated oligonucleotides. In panel A, profiles A, B and C are of oligonucleotides F, C and A, respectively after 420 minutes SVPD digestion. In panel B, profile A is of an undigested oligonucleotide phosphodiester and profile B is of the same after 1 minute SVPD digestion.

Figure 2 shows results of RNase H activation studies for oligonucleotides, as described in Example 4.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In a first aspect, the invention provides oligonucleotides that are useful for studying the parameters that are important for effective antisense oligonucleotide action. For purposes of the invention, the term oligonucleotide includes polymers of two or more ribonucleotides, deoxyribonucleotides, or both, with ribonucleotide and/or deoxyribonucleotide monomers being connected together via 5' to 3' linkages which may include any of the linkages that are known in the antisense oligonucleotide art. In addition, the term oligonucleotides includes such molecules having modified nucleic acid/bases and/or sugars, as well as such molecules having added substituents, such as diamines, cholesteryl or other lipophilic groups. Certain preferred combinations of monomers and inter-monomer linkages are discussed in greater detail below.

It is generally believed that the activity of an antisense oligonucleotide depends on the binding of the oligonucleotide to the target nucleic acid, thus disrupting the function of the target, either by hybridization arrest or by destruction of target RNA by RNase H. These mechanisms of action suggest that two parameters should be important to antisense oligonucleotide activity: duplex stability and RNase H activation. Duplex stability is important, since the oligonucleotide presumably must form a duplex (or triplex in the Hoogsteen pairing mechanism) with the target nucleic acid to act either by hybridization arrest or by RNase H-mediated target destruction. RNase H activation (the ability to activate RNase H when hybridized with target RNA) is implicated when the target nucleic acid is RNA, since such activation can lead to the effective destruction of the target RNA molecule. In addition, for an antisense oligonucleotide to act <u>in vivo</u>, it must survive long enough to interact with the target nucleic

acid. Given the fact that the in vivo environment contains endonuclease and exonuclease activities, a third parameter arises from this requirement; namely that the antisense oligonucleotide should resist nucleolytic degradation.

To analyze and explain the importance of each of these parameters to the effectiveness of antisense oligonucleotides, it is necessary to have oligonucleotides that vary in each of these parameters. The properties of several known oligonucleotides are shown in Table I, below.

TABLE I

PROPERTIES OF OLIGONUCLEOTIDES

Oligonucleotide	Duplex Stability[1]	Nuclease Resistance[2]	RNase H Activation[3]
Oligodeoxyribonucleotide (phosphate)	–	–	Yes
Oligodeoxyribonucleotide phosphorothioate	Lower	+	Yes
Oligodeoxyribonucleotide phosphorodithioate	Lower	++	Yes
Oligodeoxyribonucleotide selenoate	Lower	+	N.K.
Oligodeoxyribonucleotide phosphoramidate	Lower	+++	No
Oligoribonucleotide (phosphate)	Higher	–	No
Oligoribonucleotide phosphorothioate	Higher	+	No
2'-OMe-Oligonucleotide (phosphate)	Higher	+	No
2'-OMe-Oligoribonucleotide (phosphorothioate)	Higher	++	No
Oligodeoxyribonucleotide methylphosphonate	Lower	+++	No

1. Duplex stability of oligonucleotide to complementary oligoribonucleotide under physiological conditions, compared to DNA-RNA stability.
2. Compared from DNA (phosphodiesterase digestion).
3. Activation of RNase H by the duplex formed between oligonucleotide and RNA.

Hybrid oligonucleotides according to the invention form more stable duplexes with complementary RNA than oligodeoxyribonucleotide phosphorothioates. In addition, they are more resistant to endonucleolytic and exonucleolytic degradation than oligodeoxyribonucleotide phosphorothioates and they normally activate RNase H. Consequently, oligonucleotides according to the invention complement the oligonucleotides shown in Table I in

studies of the parameters involved in the effectiveness of antisense oligonucleotides.

With respect to this first aspect of the invention, oligonucleotides according to the invention can have any oligonucleotide sequence, since complementary oligonucleotides used in such study can be prepared having any oligonucleotide sequence. Oligonucleotides according to this aspect of the invention are characterized only by the following features. First, at least some of the internucleotide linkages present in oligonucleotides according to the invention are phosphorothioate and/or phosphorodithioate linkages. In various embodiments, the number of phosphorothioate and/or phosphorodithioate internucleotide linkages can range from 1 to as many internucleotide linkages as are present in the oligonucleotide. Thus, for purposes of the invention, the term oligonucleotide phosphorothioate and/or phosphorodithioate is intended to encompass every such embodiment. In a preferred embodiment, oligonucleotides according to the invention will range from about 2 to about 50 nucleotides in length, and most preferably from about 6 to about 50 nucleotides in length. Thus, in this preferred embodiment, oligonucleotides according to the invention will have from 1 to about 49 phosphorothioate and/or phosphorodithioate internucleotide linkages.

A second feature of oligonucleotides according to this aspect of the invention is the presence of deoxyribonucleosides. Oligonucleotides according to the invention contain at least one deoxyribonucleoside. Preferably oligonucleotides according to the invention contain four or more deoxyribonucleotides in a contiguous block, so as to provide an activating segment for RNase H. In certain preferred embodiments, more than one such activating segment will be present. Such segments may be present at any location within the oligonucleotide.

There may be a majority of deoxyribonucleosides in oligonucleotides according to the invention. In fact, such oligonucleotides may have as many as all but one nucleoside being deoxyribonucleosides. Thus, in a preferred embodiment, having from about 2 to about 50 nucleosides or most preferably from about 6 to about 50 nucleosides, the number of deoxyribonucleosides present will range from 1 to about 49 deoxyribonucleosides.

A third feature of oligonucleotides according to this aspect of the invention is the presence of ribonucleosides, 2'-substituted ribonucleosides or combinations thereof. For purposes of the invention, the term "2'-substituted" means substitution of the 2'-OH of the ribose molecule with, e.g, 2'-OMe, 2'-allyl, 2'-aryl, 2'-alkyl, 2'-halo, or 2'-amino, but not with 2'-H, wherein allyl, aryl, or alkyl groups may be unsubstituted or substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl or amino groups. Oligonucleotides according to the invention contain at least one ribonucleoside and/or 2'-substituted ribonucleoside. In a preferred embodiment, such oligonucleotides have 6 or more ribonucleosides and/or 2'-substituted ribonucleosides to enhance duplex stability. Such ribonucleosides and/or 2'-substituted ribonucleosides can be present singly, in pairs, or in larger contiguous segments, and may be present at any position within the oligonucleotide or at multiple positions within the oligonucleotide. Such ribonucleosides and/or 2'-substituted ribonucleosides may comprise as many as all but one nucleoside within the oligonucleotides. Thus, in a preferred embodiment, having from about 2 to about 50 nucleosides or most preferably from about 6 to about 50 nucleosides, the number of ribonucleosides or 2'-

substituted ribonucleosides will range from about 1 to about 49 deoxyribonucleosides.

The ability to vary the numbers and positions of phosphorothioate and/or phosphorodithioate internucleotide linkages, deoxyribonucleosides, and ribonucleosides or 2'-substituted ribonucleosides allows the investigator to examine in detail how each of these variables affects the parameters of nuclease resistance, duplex stability and RNase H activation. The ability to vary the size of the oligonucleotide allows examination of yet another parameter. In addition, smaller oligos (e.g., dimers) can be used as building blocks for larger oligos. Thus, every such possible embodiment described above is useful in such studies.

In a second aspect, the invention provides hybrid oligonucleotides that are effective in inhibiting viruses, pathogenic organisms, or the expression of cellular genes. The ability to inhibit such agents is clearly important to the treatment of a variety of disease states. Oligonucleotides according to this aspect of the invention share the characteristics of the above-described oligonucleotides, except that the oligonucleotide sequence of oligonucleotides according to this aspect of the invention is complementary to a nucleic acid sequence that is from a virus, a pathogenic organism or a cellular gene. Preferably such oligonucleotides are from about 6 to about 50 nucleotides in length. For purposes of the invention, the term "oligonucleotide sequence that is complementary to a nucleic acid sequence" is intended to mean an oligonucleotide sequence (2 to about 50 nucleotides) that hybridizes to the nucleic acid sequence under physiological conditions, e.g. by Watson-Crick base pairing (interaction between oligonucleotide and single-stranded nucleic acid) or by Hoogsteen base pairing

(interaction between oligonucleotide and double-stranded nucleic acid) or by any other means. Such hybridization under physiological conditions is measured as a practical matter by observing interference with the function of the nucleic acid sequence.

The nucleic acid sequence to which an oligonucleotide according to the invention is complementary will vary, depending upon the agent to be inhibited. In many cases the nucleic acid sequence will be a virus nucleic acid sequence. The use of antisense oligonucleotides to inhibit various viruses is well known, and has recently been reviewed in Agrawal, Tibtech 10:152-158 (1992). Viral nucleic acid sequences that are complementary to effective antisense oligonucleotides have been described for many viruses, including human immunodeficiency virus type 1 (U.S. Patent No. 4,806,463, the teachings of which are herein incorporated by reference), Herpes simplex virus (U.S. Patent No. 4,689,320, the teachings of which are hereby incorporated by reference), Influenza virus (U.S. Patent No. 5,XXX,XXX; Ser. No. 07/516,275, allowed June 30, 1992; the teachings of which are hereby incorporated by reference), and Human papilloma virus (Storey et al., Nucleic Acids Res. 19:4109-4114 (1991)). Sequences complementary to any of these nucleic acid sequences can be used for oligonucleotides according to the invention, as can be oligonucleotide sequences complementary to nucleic acid sequences from any other virus. Additional viruses that have known nucleic acid sequences against which antisense oligonucleotides can be prepared include Foot and Mouth Disease Virus (See Robertson et al., J. Virology 54: 651 (1985); Harris et al., J. Virology 36: 659 (1980)), Yellow Fever Virus (See Rice et al., Science 229: 726 (1985)), Varicella-Zoster Virus (See Davison and Scott, J. Gen. Virology 67: 2279 (1986), and Cucumber

Mosaic Virus (See Richards et al., Virology 89: 395 (1978)).

Alternatively, oligonucleotides according to the invention can have an oligonucleotide sequence complementary to a nucleic acid sequence of a pathogenic organism. The nucleic acid sequences of many pathogenic organisms have been described, including the malaria organism, Plasmodium falciparum, and many pathogenic bacteria. Oligonucleotide sequences complementary to nucleic acid sequences from any such pathogenic organism can be used in oligonucleotides according to the invention. Examples of pathogenic eukaryotes having known nucleic acid sequences against which antisense oligonucleotides can be prepared include Trypanosoma brucei gambiense and Leishmania (See Campbell et al., Nature 311: 350 (1984)), Fasciola hepatica (See Zurita et al., Proc. Natl. Acad. Sci. USA 84: 2340 (1987). Antifungal oligonucleotides can be prepared using a target hybridizing region having an oligonucleotide sequence that is complementary to a nucleic acid sequence from, e.g., the chitin synthetase gene, and antibacterial oligonucleotides can be prepared using, e.g., the alanine racemase gene.

In yet another embodiment, the oligonucleotides according to the invention can have an oligonucleotide sequence complementary to a cellular gene or gene transcript, the abnormal expression or product of which results in a disease state. The nucleic acid sequences of several such cellular genes have been described, including prion protein (Stahl and Prusiner, FASEB J. 5: 2799-2807 (1991)), the amyloid-like protein associated with Alzheimer's disease (U.S. Patent No. 5,015,570, the teachings of which are hereby incorporated by reference), and various well-known oncogenes and proto-oncogenes, such as c-myb, c-myc, c-abl, and n-ras. In addition,

oligonucleotides that inhibit the synthesis of structural proteins or enzymes involved largely or exclusively in spermatogenesis, sperm motility, the binding of the sperm to the egg or any other step affecting sperm viability may be used as contraceptives for men. Similarly, contraceptives for women may be oligonucleotides that inhibit proteins or enzymes involved in ovulation, fertilization, implantation or in the biosynthesis of hormones involved in those processes.

Hypertension can be controlled by oligodeoxynucleotides that suppress the synthesis of angiotensin converting enzyme or related enzymes in the renin/angiotensin system; platelet aggregation can be controlled by suppression of the synthesis of enzymes necessary for the synthesis of thromboxane A2 for use in myocardial and cerebral circulatory disorders, infarcts, arteriosclerosis, embolism and thrombosis; deposition of cholesterol in arterial wall can be inhibited by suppression of the synthesis of fattyacryl co-enzyme A: cholesterol acyl transferase in arteriosclerosis; inhibition of the synthesis of cholinephosphotransferase may be useful in hypolipidemia.

There are numerous neural disorders in which hybridization arrest can be used to reduce or eliminate adverse effects of the disorder. For example, suppression of the synthesis of monoamine oxidase can be used in Parkinson's disease; suppression of catechol o-methyl transferase can be used to treat depression; and suppression of indole N-methyl transferase can be used in treating schizophrenia.

Suppression of selected enzymes in the arachidonic acid cascade which leads to prostaglandins and leukotrienes may be useful in the control of platelet aggregation, allergy, inflammation, pain and asthma.

Suppression of the protein expressed by the multidrug resistance (mdr) gene, which is responsible for

development of resistance to a variety of anti-cancer drugs and is a major impediment in chemotherapy may prove to be beneficial in the treatment of cancer.

Oligonucleotide sequences complementary to nucleic acid sequences from any of these genes can be used for oligonucleotides according to the invention, as can be oligonucleotide sequences complementary to any other cellular gene or gene transcript, the abnormal expression or product of which results in a disease state.

Antisense regulation of gene expression in plant cells has been described in U.S. Patent No. 5,107,065, the teachings of which are hereby incorporated by reference.

In a third aspect, the invention provides therapeutic pharmaceutical formulations of oligonucleotides that are effective for treating virus infection, infections by pathogenic organisms, or disease resulting from abnormal gene expression or from the expression of an abnormal gene product. Such therapeutic pharmaceutical formulations comprise the oligonucleotides according to the second aspect of the invention in a pharmaceutically acceptable carrier.

In a fourth aspect, the invention provides a method for inhibiting the gene expression of a virus, a pathogenic organism or a cellular gene, the method comprising the step of providing oligonucleotides according to the invention to cells infected with the virus or pathogenic organism in the former two cases or to cells generally in the latter case. Such methods are useful in studying gene expression and the function of specific genes.

In a fifth aspect, the invention provides a method of treating a diseased human or animal in which the

disease results from infection with a virus or pathogenic organism, or from the abnormal expression or product of a cellular gene. The method comprises administering therapeutic pharmaceutical formulations of oligonucleotides according to the invention to the diseased human or animal. Preferably, the routes of such administration will include oral, intranasal, rectal and topical administration. In such methods of treatment according to the invention the oligonucleotides may be administered in conjunction with other therapeutic agents, e.g., AZT in the case of AIDS.

A variety of viral diseases may be treated by the method of treatment according to the invention, including AIDS, ARC, oral or genital herpes, papilloma warts, flu, foot and mouth disease, yellow fever, chicken pox, shingles, HTLV-leukemia, and hepatitis. Among fungal diseases treatable by the method of treatment according to the invention are candidiasis, histoplasmosis, cryptococcocis, blastomycosis, aspergillosis, sporotrichosis, chromomycosis, dermatophytosis and coccidioidomycosis. The method can also be used to treat rickettsial diseases (e.g., typhus, Rocky Mountain spotted fever), as well as sexually transmitted diseases caused by Chlamydia trachomatis or Lymphogranuloma venereum. A variety of parasitic diseases can be treated by the method according to the invention, including amebiasis, Chegas' disease, toxoplasmosis, pneumocystosis, giardiasis, cryptosporidiosis, trichomoniasis, and Pneumocystis carini pneumonia; also worm (helminthic diseases) such as ascariasis, filariasis, trichinosis, schistosomiasis and nematode or cestode infections. Malaria can be treated by the method of treatment of the invention regardless of whether it is caused by P. falciparum, P. vivax, P. orale, or P. malariae.

The infectious diseases identified above can all be treated by the method of treatment according to the invention because the infectious agents for these diseases are known and thus oligonucleotides according to the invention can be prepared, having oligonucleotide sequence that is complementary to a nucleic acid sequence that is an essential nucleic acid sequence for the propagation of the infectious agent, such as an essential gene.

Other disease states or conditions that are treatable by the method according to the invention result from an abnormal expression or product of a cellular gene. These conditions can be treated by administration of oligonucleotides according to the invention., and have been discussed earlier in this disclosure.

Oligonucleotides according to the invention can be synthesized by procedures that are well known in the art. Alternatively, and preferably such oligonucleotides can be synthesized by the H-phosphonate approach described in U.S. Patent No. 5,XXX,XXX (Ser. No. 07/334,679; allowed on March 10, 1992), the teachings of which are hereby incorporated by reference, and in Agrawal and Tang, Tetrahedron Lett. 31: 7541-7544 (1990). Oligonucleotides according to the invention can be made even more resistant to nucleolytic degradation through the addition of cap structures at the 5' and/or 3' end.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not intended to be limiting in nature.

Example 1

Synthesis of Hybrid Oligonucleotide Phosphorothioates

Hybrid oligonucleotide phosphorothioates were synthesized on CPG on a 5-6 μmole scale on an automated synthesizer (model 8700, Millipore, Milford, MA) using the H-phosphonate approach described in U.S. Patent No. 5,XXX,XXX (Ser. No. 07/344,679; allowed on March 19, 1992). Deoxynucleoside H-phosphonates were obtained from Millipore. 2'-OMe ribonucleotide H-phosphonates were synthesized by standard procedures. Segments of oligonucleotides containing 2'-OMe nucleoside were assembled by using 2'-OMe ribonucleoside H-phosphonates for the desired cycles. Similarly, segments of oligonucleotides containing deoxyribonucleosides were assembled by using deoxynucleoside H-phosphonates for the desired cycles. After assembly, CPG bound oligonucleotide H-phosphonate was oxidized with sulfur to generate the phosphorothioate linkage. Oligonucleotides were then deprotected in concentrated NH_4OH at 40°C for 48 hours.

Crude oligonucleotide (about A_{260} units) was analyzed on reverse low pressure chromatography on a C_{18} reversed phase medium. The DMT group was removed by treatment with 80% aqueous acetic acid, then the oligonucleotides were dialyzed against distilled water and lyophilized.

The oligonucleotides synthesized are shown in Table II, below.

TABLE II

HYBRID OLIGONUCLEOTIDE PHOSPHOROTHIOATES* SYNTHESIZED

Oligo	Structure
A	5'A C A C C C A A T T C T G A A A A T G G 3'
B	A C A C C C A A T T C U G A A A A U G G
C	A C A C C C A A T T C T G A A A A U G G
D	A C A C C C A A T T C U G A A A A U G G
E	A C A C C C A A U T C T G A A A A T G G
F	A C A C C C A A U U C U G A A A A U G G

Underlined sequences contain 2'-OMe ribonucleoside.
* All internucleotide linkages are phosphorothioate linkages for oligos A-G.

Example 2
Relative Nuclease Resistance of Hybrid Oligonucleotide Phosphorothioates

To test the relative nuclease resistance of various hybrid oligonucleotide phosphorothioates, the oligonucleotides were treated with snake venom phosphodiesterase (SVPD). About 0.2 A_{260} units of oligos A, C and F were dissolved in 500µl buffer (40mM NH_4CO_3, pH 0.4 + 20mM $MgCl_2$) and mixed with _____ units SVPD. The mixture was incubated at 37°C for 420 minutes. After 0, 200 and 420 minutes, 165µl aliquots were removed and analyzed using ion exchange HPLC. The results are shown in Figure 1. Oligonucleotide F was very resistant to phosphodiesterase, whereas oligonucleotide A was digested almost to completion and oligonucleotide C was digested to 50% (panel A). An oligonucleotide phosphodiester was

digested to about 80% in one minute using one tenth of the concentration of SVPD.

These results indicate that the presence of 2'-OMe ribonucleosides in an oligonucleotide phosphorothioate enhances resistance to exonucleolytic digestion and that this enhanced resistance increased when a larger proportion of 2'-OMe ribonucleotides are used. Due to the similar character and behavior of ribonucleotides, other 2'-substituted ribonucleotides and 2'-OMe ribonucleotides, these results also suggest that similar enhancement of nuclease resistance would be obtained for hybrid oligonucleotide phosphorothioates and/or phosphorodithioates having ribonucleotides, 2'-substituted ribonucleotides, or a mixture of ribonucleotides and 2'-substituted ribonucleotides.

Example 3

Relative Duplex Stability of Hybrid Oligonucleotide Phosphorothioates

Oligonucleotides A-F were tested for their relative stability of duplexes formed with complementary oligodeoxyribonucleotides, and with complementary oligoribonucleotides. In separate reactions, each oligonucleotide A-F was mixed with an equivalent quantity (0.2 A_{260} units) of its complementary oligonucleotide in 150 mM NaCl, 10mM Na_2PO_4, 1mM EDTA, pH 7. The mixture was heated to 85°C for 5 minutes, then cooled to 30°C. The temperature was then increased from 30°C to 80°C at a rate of 1°C per minute and A_{260} was recorded as a function of temperature. The results are shown in Table III, below.

TABLE III

MELTING TEMPERATURE OF DUPLEXES

Oligonucleotide Number	DNA-DNA Duplex		DNA-RNA Duplex		Tm RNA Duplex-Tm RNA Duplex (°C)	DNA-RNA Duplex w/Magnesium	
	Tm DNA (°C)	Difference in Tm compared to oligonucleo-tide A(°C)	Tm RNA (°C)	Difference in Tm compared to oligonucleo-tide A(°C)		Tm RNA (Mg)(°C)	Difference in Tm compared to oligonucleo-tide A(°C)
A	51.1	0	43.4	0	-7.7	48.1	0
B	48.3	-2.8	50.9	7.5	2.6	58.4	10.3
C	49.9	-1.2	48.9	5.5	-1.0	54.2	6.1
D	45.1	-6.0	50.9	7.5	5.8	56.1	8.0
E	47.2	-3.9	51.1	7.7	3.9	56.5	8.4
F	47.6	-3.5	61.1	17.7	13.5	69.1	21.0

These results reveal that when the complementary oligonucleotide is an oligoribonucleotide, the presence of 2'-OMe ribonucleotides enhances duplex stability, and that this enhancement increases with increased proportions of 2'-OMe ribonucleosides. These results should be similarly applicable to hybrid oligonucleotide phosphorothioates and/or phosphorodithioates containing ribonucleotides, 2'-substituted ribonucleotides, or mixtures of ribonucleotides and 2'-substituted ribonucleotides. Thus, the hybrid oligonucleotide phosphorothioates and/or phosphorodithioates according to the invention should bind viral RNA or virus, pathogenic organism or cellular mRNA with greater affinity than ordinary oligodeoxynucleotide phosphorothioates.

Example 4

Activation of RNase H by Hybrid Oligonucleotide Phosphorothioates

Oligonucleotide phosphorothioates and various hybrid oligonucleotide phosphorothioates were studied for their RNase H activation properties. Oligonucleotide A (Table II), an oligonucleotide phosphorothioate which is known to activate RNase H, was used as a control. Oligonucleotide F (a 2'-OMe analog of oligonucleotide phosphorothioate) and oligonucleotides C, B, and E, hybrid oligonucleotides, were studied for their ability to activate RNase H.

To carry out the experiment, a complementary 32-mer oligoribonucleotide was synthesized (Figure 2) and kinased at the 5'-end, ^{32}P-labeled 32-mer RNA (0.003 A_{260} units; 0.01 μg) and oligonucleotides (0.0635 A_{260} units; 1.9 μg) were mixed in the 20 μl of buffer (0.15 M NaCl, 0.01 $MgCl_2$, 0.01 M Tris chloride, pH 7.9, containing 0.001 M DTT. The mixture was incubated with 6 units of RNase H (*E. Coli*) at 37°C. Aliquots of 4.5 μl were removed at 0, 15, 30, and 60 minutes and analyzed on polyacrylamide gel electrophoresis.

Oligonucleotide A (Duplex A) showed site specific cleavage of RNA by RNase H. Oligonucleotide F (2'-OMe analog; Duplex B) showed no cleavage of RNA in presence of RNase H. Hybrid oligonucleotide B, C, and E (Duplexes C, D, E, resp.) showed site specific cleavage of RNA by RNase H. Duplex F, in which a mismatched oligonucleotide phosphorothioate was studied showed no cleavage of RNA. Lane G shows that in presence of RNase H, RNA was not cleaved.

Example 5

Inhibition of HIV by Hybrid Oligonucleotide Phosphorothioates

Hybrid oligonucleotide phosphorothioates were tested for their ability to inhibit HIV-1 in tissue culture. H9 lymphocytes were infected with HIV-1 virions (≡0.01 - 0.1 $TCID_{50}$/cell) for one hour at 37°C. After one hour, unadsorbed virions were washed and the infected cells were divided among wells of 24 well plates. To the infected cells, an appropriate concentration (from stock solution) of oligonucleotide was added to obtain the required concentration in 2 ml medium. In a positive control experiment ddC or AZT was added. The cells were then cultured for three days. At the end of three days, supernatant from the infected culture was collected and measured for p24 expression by ELISA. The level of expression of -24 was compared between oligonucleotide treated and untreated (no drug) infected cells.

All of the hybrid oligonucleotide phosphorothioates tested showed significant inhibition of p24 expression at µg/ml concentrations, without significant cytotoxicity (data not shown). These results indicate that hybrid oligonucleotide phosphorothioates containing 2'-OMe ribonucleotides are effective as inhibitors of gene

expression. Similar effectiveness would be expected for hybrid oligonucleotide phosphorothioates and/or phosphorodithioates containing ribonucleosides, 2'-substituted ribonucleosides, or a mixture of ribonucleosides and 2'-substituted ribonucleosides.

WE CLAIM:

1. A hybrid oligonucleotide phosphorothioate and/or phosphorodithioate comprising one each of the following: a deoxyribonucleoside, a ribonucleoside or a 2'-substituted ribonucleoside, and a phosphorothioate and/or phosphorodithioate internucleotide linkage.

2. A hybrid oligonucleotide phosphorothioate and/or phosphorodithioate according to claim 1, wherein the deoxyribonucleoside is present in a segment of at least four contiguous deoxyribonucleosides.

3. A hybrid oligonucleotide phosphorothioate and/or phosphorodithioate according to claim 1, wherein the ribonucleoside or 2'-substituted ribonucleoside is present in a segment of at least two contiguous ribonucleosides and/or 2'-substituted ribonucleosides.

4. A hybrid oligonucleotide phosphorothioate and/or phosphorodithioate according to claim 1, having an oligonucleotide sequence that is complementary to a nucleic acid sequence from a virus, a pathogenic organism, or a cellular gene.

5. A hybrid oligonucleotide phosphorothioate and/or phosphorodithioate according to claim 2, having an oligonucleotide sequence that is complementary to a nucleic acid sequence from a virus, a pathogenic organism, or a cellular gene.

6. A hybrid oligonucleotide phosphorothioate and/or phosphorodithioate according to claim 3, having an oligonucleotide sequence that is complementary to a nucleic acid sequence from a virus, a pathogenic organism, or a cellular gene.

7. A therapeutic pharmaceutical formulation comprising an oligonucleotide according to claim 4 in a pharmaceutically acceptable carrier.

8. A therapeutic pharmaceutical formulation comprising an oligonucleotide according to claim 5 in a pharmaceutically acceptable carrier.

9. A therapeutic pharmaceutical formulation comprising an oligonucleotide according to claim 6 in a pharmaceutically acceptable carrier.

10. A method of inhibiting the gene expression of a virus, a pathogenic organism, or a cellular gene, the method comprising the step of providing an oligonucleotide according to claim 4 to a cell that is infected with a virus, to a pathogenic organism, or to a cell, respectively.

11. A method of inhibiting the gene expression of a virus, a pathogenic organism, or a cellular gene, the method comprising the step of providing an oligonucleotide according to claim 5 to a cell that is infected with a virus, to a pathogenic organism, or to a cell, respectively.

12. A method of inhibiting the gene expression of a virus, a pathogenic organism, or a cellular gene, the method comprising the step of providing an oligonucleotide according to claim 6 to a cell that is infected with a virus, to a pathogenic organism, or to a cell, respectively.

FIGURE 1a

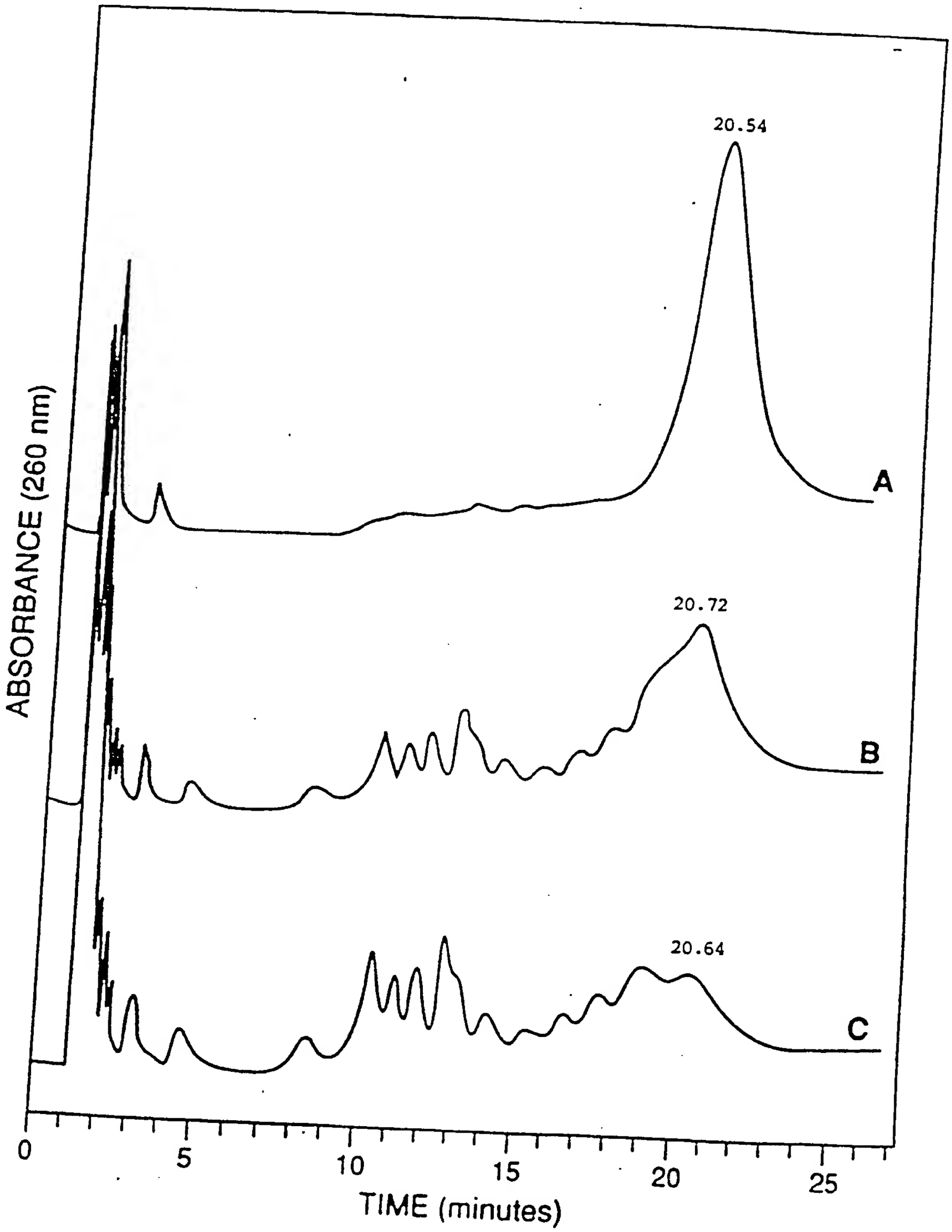

20.54
A
20.72
B
20.64
C
ABSORBANCE (260 nm)
0
5
10
15
20
25
TIME (minutes)

FIGURE 1b

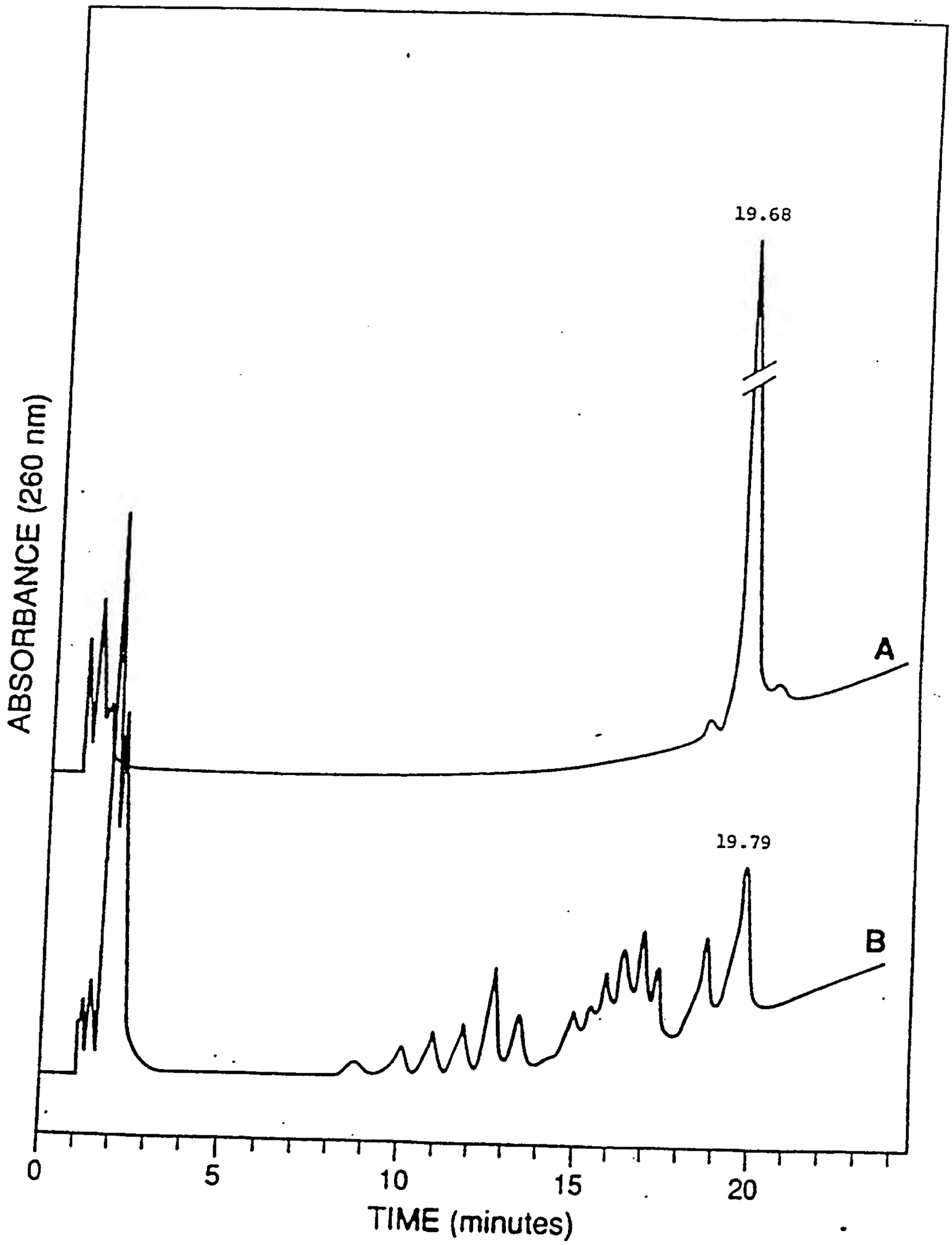
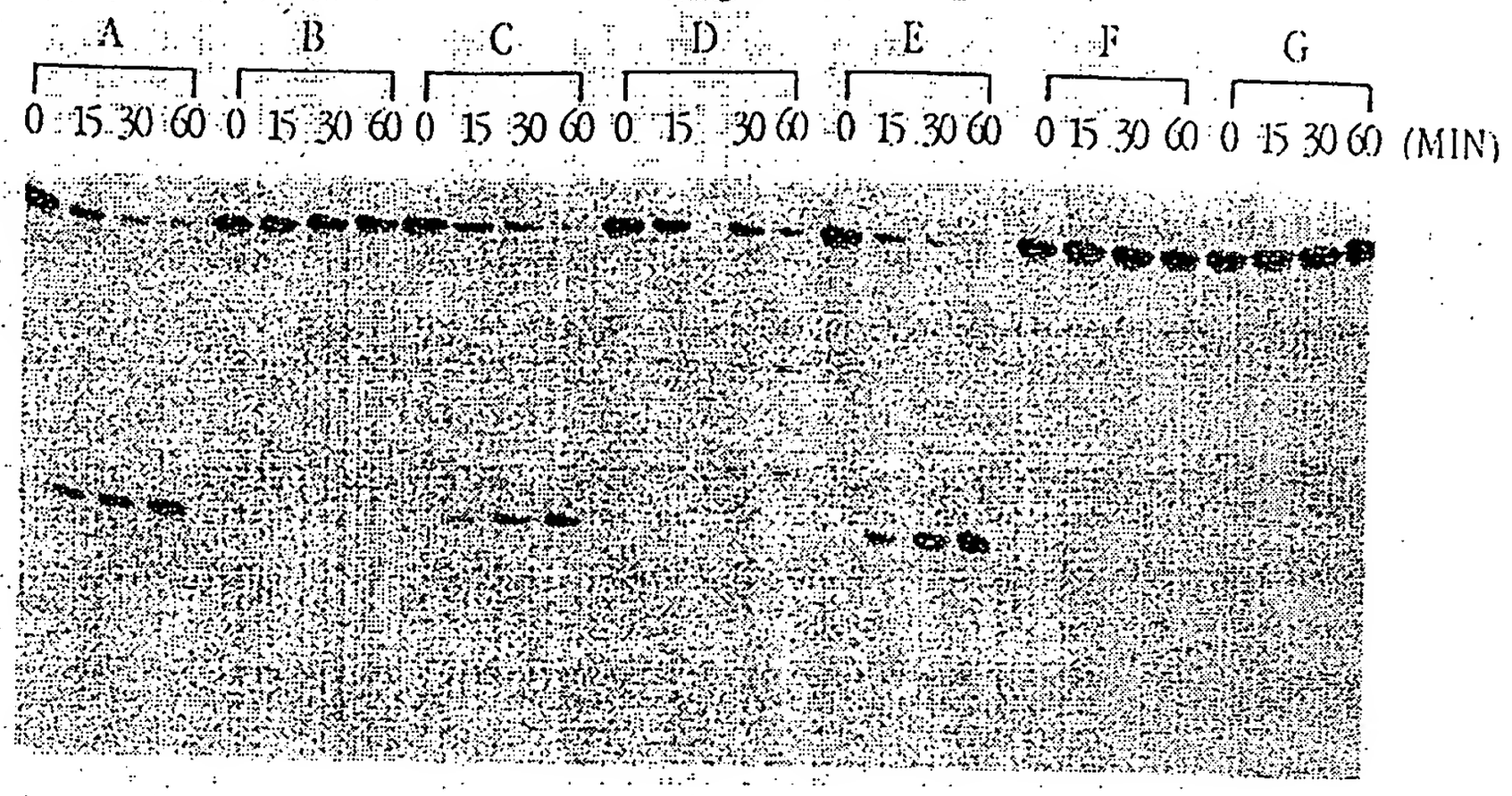


Figure 2



		Oligo-nucleotide	Du-plex
RNA 3′ UACAGCUGUGGGUUAAGACUUUUACCUAUUUG 5′ 5′ ACACCCAATTCTGAAAATGG 3′		A	1
RNA 3′ UACAGCUGUGGGUUAAGACUUUUACCUAUUUG 5′ 5′ ACACCCAAUUCUGAAAAUGG 3′		F	2
RNA 3′ UACAGCUGUGGGUUAAGACUUUUACCUAUUUG 5′ 5′ ACACCCAATTCTGAAAAUGG 3′		C	3
RNA 3′ UACAGCUGUGGGUUAAGACUUUUACCUAUUUG 5′ 5′ ACACCCAATTCUGAAAAUGG 3′		B	4
RNA 3′ UACAGCUGUGGGUUAAGACUUUUACCUAUUUG 5′ 5′ ACACCCAAUTCTGAAAATGG 3′		E	5
RNA 3′ UACAGCUGUGGGUUAAGACUUUUACCUAUUUG 5′ 5′ ACAGACTTACCTCAGATAAT 3′		MIS-MATCHED	6
RNA 3′ UACAGCUGUGGGUUAAGACUUUUACCUAUUUG 5′		—	—
= 2′ -OCH_3 -RIBO UNITS			

INTERNATIONAL SEARCH REPORT

Int sional Application No
PCT/US 93/06884

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07H21/00 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07H A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TRENDS IN BIOTECHNOLOGY vol. 10, no. 5 , May 1992 , CAMBRIDGE GB pages 152 - 158 AGRAWAL S. 'Antisense oligonucleotides as antiviral agents' cited in the application see table 2 ---	1-12
Y	WO,A,90 15814 (MEIOGENICS INC) 27 December 1990 see the whole document ---	1-12
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 November 1993	30. 11. 93

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	DAY, G

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INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 93/06884

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NUCLEIC ACIDS RESEARCH vol. 19, no. 11 , 1991 , ARLINGTON, VIRGINIA US pages 2979 - 2986 KIBLER-HERZOG L. ET AL 'Duplex stabilities of phosphorothioate, methylphosphonate, and RNA analogs of two DNA 14-mers' see abstract	1-12
Y	NUCLEIC ACIDS RESEARCH vol. 17, no. 1 , 1989 , ARLINGTON, VIRGINIA US pages 239 - 252 SHIBAHARA S. ET AL 'Inhibition of human immunodeficiency virus (HIV-1) replication by synthetic oligo-RNA derivatives' see abstract	1-12
Y	FEBS LETTERS vol. 215, no. 2 , May 1987 , AMSTERDAM NL pages 327 - 330 INOUE H ET AL 'Sequence-dependent hydrolysis of RNA using modified oligonucleotide splints and RNase H' cited in the application see the whole document	1-12

INTERNATIONAL SEARCH REPORT

Int. ..ational application No.
PCT/US93/06884

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although ~~claims~~ claims 10-12 are partially directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/US 93/06884

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9015814	27-12-90	AU-A- 5931290	08-01-91